1083

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[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

Phosphopeptides Obtained by Partial Acid Hydrolysis of α-Casein[2]

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A partial acid hydrolysis of α-casein was resolved into fractions containing phosphopeptides by chromatography on Dowex 50. The impure fractions were further purified by chromatography and the electrophoretically pure subfractions were analyzed for their amino acid composition on Dowex 50. In addition to phosphoserine the following dipeptides were found: phosphoserylglutamic acid, phosphoserylalanine and phosphoserylphosphoserine. The extensive destruction of serine on complete hydrolysis of the peptides is discussed.

The physiological significance of phosphorus in casein and other phosphoproteins has been the subject of a number of recent investigations.[3] The presence of phosphorus is also advantageous in structural studies on casein since partial hydrolysis with enzymes and acid produces phosphopeptides which can be uniquely separated from the non-phosphopeptides. Numerous phosphopeptide fractions have been prepared from casein, particularly by enzymic digestion.[4] Lipmann[5] has shown that at least 50% of the casein phosphorus is present as ser-

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(3) (a) O. Mellander, *Acta Soc. Med. Upsaliensis*, **55**, 247 (1950); (b) O. Mellander and B. Isaksson, *ibid.*, **55**, 239 (1950); (c) E. P. Kennedy and S. W. Smith, *J. Biol. Chem.*, **207**, 153 (1954).

(4) (a) T. Posternak and S. Grafl, *Helv. Chim. Acta*, **28**, 1258 (1945); (b) C. Rimington and H. D. Kay, *Biochem. J.*, **20**, 777 (1926); (c) P. A. Levene and D. W. Hill, *J. Biol. Chem.*, **101**, 711 (1933); (d) B. H. Nicolet and L. A. Shinn, Abstracts of Papers, 110th Meeting Am. Chem. Soc., 1946, 20B; (e) M. Damodaran and B. V. Ramachandran, *Biochem. J.*, **35**, 122 (1941); (f) O. Mellander, *Upsala Läkarefören. Förd.*, **52**, 107 (1947).

(5) F. Lipmann, *Biochem. Z.*, **262**, 3 (1933).

ine phosphate after partial acid hydrolysis. de Verdier[6] has isolated small amounts of phosphothreonine from casein. Methods have been described by de Verdier[6] and Ågren and Glomset[7] for the fractionation of phosphopeptides from partial hydrolysates of casein.

The present study describes the separation and composition of some of the phosphopeptides produced by partial acid hydrolysis of α-casein

Experimental

Partial Acid Hydrolysis of Casein.—Exploratory experiments established that heating at 80° for 10 hours in 2.2 *N* hydrochloric acid was to be preferred for preparing phosphopeptides with minimum formation of inorganic phosphorus. These conditions were observed, using a 3-g. sample of finely divided α-casein prepared by the urea method[8] suspended in 360 ml. of hydrochloric acid. At the end of hydrolysis the solution was light brown and contained a small amount of insoluble material. Hydrochloric acid was removed at 25–30° with the Craig rotary evaporator.[9] Evaporation was repeated three times after the addition of water. The residue was then extracted with 0.05 *N* hydrochloric acid and the insoluble material removed by centrifugation. The soluble portion was made to 100-ml. volume. Under these conditions of hydrolysis, 40% of the peptide bonds of α-casein were broken as was shown by Van Slyke amino nitrogen analyses. The insoluble material accounted for 10% of the original nitrogen or phosphorus and was probably essentially unchanged α-casein.

Phosphoserine and phosphotyrosine used for identity purposes, were made by the method of Levene and Schormüller[10] as modified by Plimmer.[11] The analysis and properties of the phosphoserine have previously been reported.[12] The sample of synthetic phosphothreonine was kindly furnished by Dr. Bernard J. Jandorf of the Army Chemical Center, Maryland.

Color values for the phospho-amino acids on a molar basis relative to leucine using the photometric ninhydrin method of Moore and Stein[13] were as follows: phosphoserine 1.03, phosphotyrosine and phosphothreonine 0.94. With a Dowex 50-X8,[14] 120 mesh (see below), 0.9 × 100 cm. resin column using 0.05 *N* hydrochloric acid as influent as used by Schaffer, *et al.*,[15] phosphoserine and phosphothreonine are resolved into sharp peaks at 92 and 163 ml., respectively, and phosphotyrosine is resolved into a broader peak at 460 ml.

Ion-exchange Chromatography.—Dowex 50-X8 resin, 200–400 mesh, was treated with hydrochloric acid and 1 *N* sodium hydroxide as described by Moore and Stein.[16] The resin used for the separation of the phosphopeptides was passed through a 120-mesh screen and that used for the determination of amino acids through a 200-mesh screen. The hydrogen form of the resin that was used for the separation of phosphopeptides was obtained by washing the sodium form in a column with 1 *N* hydrochloric acid until the effluent was free of salt as determined by evaporation of 200-ml. aliquots. It was found that the completeness of the removal of sodium ion from the resin had a pronounced effect on the resolution of the phosphopeptides on elution with dilute hydrochloric acid. All of the resin columns were poured in at least five sections with a slurry of the resin in the first influent solvent and at least five hold-up volumes were run through the column before it was charged. A column 0.9 × 100 cm. was used for the exploratory runs of acid hydrolysates and a column 3.8 × 100 cm. for the 3-g. hydrolysate. Sufficient pressure was applied to the column to give a flow rate of 4 ml. per hour with the smaller columns and 50 ml. per hour for the large columns. Fractions of 1 ml. were collected from the small column and 8.5 ml. from the large column with an automatic fraction collector. The location of the phosphopeptides was determined by the photometric ninhydrin method of Moore and Stein[13] using 0.5-ml. aliquots from the large columns. The location of the inorganic phosphorus was determined by spot tests[17] and estimated by the method of Sumner.[18]

The Dowex 1-X10 resin, 200–400 mesh, was washed on a fritted glass Buchner funnel with 500 ml. of 2 *N* sodium hydroxide using slight suction. After washing with water followed by 1 *N* hydrochloric acid and then water, the resin was suspended in about 5 times its volume of water and after standing 2 hours the supernatant was decanted. The last step was repeated about 5 times until the final wash was almost clear. The resin was then passed through a 120-mesh screen.

Paper Chromatography.—Two-dimensional paper chromatograms for the qualitative determination of amino acids were prepared by the descending method as described by Levy and Chung[19] using butanol–acetic acid–water (4:1:5) and buffered phenol.

For the determination of the homogeneity of phosphopeptide fractions one-dimensional chromatograms using the butanol–acetic acid–water solvent system has been found to be useful. For the slow-moving acidic phosphopeptides the best results are obtained by running the chromatogram for more than 24 hours and allowing the solvent to drip from the serrations at the end of the paper.

Paper Electrophoresis.—The method described by Kunkel and Tiselius[20] with Whatman 3-MM paper between glass plates was used. The best results were obtained by performing the experiments in a cold room at 4°. A potential of 400 volts was applied to the paper between glass plates 15 × 45.5 cm. using 0.1 *N* acetic acid as the buffer. For paper electrophoresis of the phosphopeptides the use of acetic or formic acid as buffer gave satisfactory results. No satisfactory resolution of phosphopeptides was obtained when Veronal was used as the buffer at *p*H 8.6.

N-Terminal Group Analysis.—The peptides were treated with the Sanger reagent,[21] 1-fluoro-2,4-dinitrobenzene (DNFB), as described by Davie and Neurath.[22] The DNP peptides were hydrolyzed with constant boiling hydrochloric acid in sealed tubes for 8 hours at 105° since Levy and Li[23] have found that 90% of the DNP terminal serine is destroyed when DNP-ACTH is hydrolyzed for 24 hours at 110° with constant boiling hydrochloric acid. The ether-soluble extracts of the hydrolyzed DNP peptides were then chromatographed on paper with 1.5 *M* phosphate buffer at *p*H 6.0 as described by Levy.[24] The colorless water-soluble fractions were chromatographed on paper with butanol–acetic acid–water.[19] The DNP amino acids used for control substances were prepared and described by Mellon, *et al.*[25]

Amino Acid Content of Phosphopeptides.—The amino acid composition of the phosphopeptides was determined by the Moore and Stein method[16] using Dowex 50-X8 resin. The peptides were hydrolyzed with 6 *N* hydrochloric acid at 120° for 20, 70 and 140 hours. The values for the serine content of the 70- and 140-hour hydrolysates were extrapolated to zero time to determine the initial serine content. A chromatogram of a 20-hour hydrolysate of phosphopeptide 4-C, which are the conditions frequently used for complete hydrolysis of protein, clearly showed incomplete hydrolysis

(6) C. H. de Verdier, *Nature*, **170**, 804 (1952).

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(14) Mention of products in this paper does not imply endorsement or recommendation by the Department of Agriculture over similar products not mentioned.

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(16) S. Moore and W. H. Stein, *ibid.*, **192**, 663 (1951).

(17) F. Feigl, "Laboratory Manual of Spot Tests," Academic Press, Inc., New York, N.Y., 1943, p. 146.

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(21) F. Sanger and E. O. P. Thompson, *Biochem. J.*, **53**, 353 (1953).

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(23) A. L. Levy and C. H. Li, *ibid.*, **213**, 487 (1955).

(24) A. L. Levy, *Nature*, **174**, 126 (1954).

(25) E. F. Mellon, A. H. Korn and S. R. Hoover, THIS JOURNAL, **75**, 1675 (1953).

of the organic phosphorus as well as of the peptide. This was confirmed by the chromatogram of the 70-hour hydrolysate where the peak due to phosphoserine could not be detected and the serine and glutamic acid content increased. Differences in the rate of destruction of serine in the hydrolysis of various proteins are discussed by Smith and Stockell.[26] They predict, on the basis of the reports by Sanger[27] and Leach,[28] that serine, threonine and aspartic acid will be liberated completely from peptides after 20 hours of hydrolysis. It is apparent, however, that complete hydrolysis of phosphoserine and its peptides is not obtained in 20 hours. Rees[29] has shown that the destruction of serine on hydrolysis is accompanied by the formation of ammonia. Smith and Stockell[26] have shown that for the hydrolysis of papain and carboxypeptidase the increase in ammonia parallels the destruction of serine, but that the percentage loss of serine at a given time depends on the initial amino acid content of the protein, as well as on unknown factors. For the determination of the destruction of serine on hydrolysis of phosphoseryl peptides, ammonia was determined with a short column (0.9 × 15 cm.) of Dowex 50-X8 as described by Moore and Stein[16] using only the *p*H 5.0 and *p*H 6.5 citrate buffers, since basic amino acids were not present.

Results

Chromatography of Phosphopeptides on Dowex 50-X8.—The partial acid hydrolysate from 3 g. of α-casein was made to 100 ml. as previously described. Seventy-five milliliters, amounting to 2.25 g. of protein, was chromatographed on a 3.8 × 100 cm. column of Dowex 50-X8. The results for two hydrolysates are shown in Fig. 1. The chromatograms show that the hydrolysis and resolution into phosphopeptide fractions are highly reproducible. The nitrogen and phosphorus content of these fractions are given in Table I. Under the conditions employed the non-phosphorus portion of the hydrolysate is retained by the resin. Of the total phosphorus put on the column 90.7% was eluted and is associated with only 8.9% of the nitrogen. The relatively high amount of nitrogen and phosphorus in the base-line fractions is due in part to the large number of tubes with low optical density that were not satisfactorily resolved, and possibly in part to the low color values of the components. All of the inorganic phosphorus that was formed on hydrolysis, equivalent to only 9.35% of the phosphorus of α-casein subjected to hydrolysis, is eluted in fraction 2, near the front of the chromatogram, together with peptides containing organic phosphorus. It is of interest to note that the phosphopeptides in fraction 1 have less affinity for the resin than does inorganic phosphorus. The total organic phosphorus in the six fractions, equivalent to 56% of the phosphorus put on the column, is associated with 5.1% of the nitrogen put on the column. The atomic ratios of N–P of the fractions reported in the last column of Table I are higher than they should be since they contain free ammonia; the uptake of ammonia by the acid solution during the concentration and handling of the fractions is difficult to prevent. The low ratios of nitrogen to phosphorus, however, reflect the concentration of phosphorus when compared to α-casein with a ratio of 34.4.

(26) E. L. Smith and A. Stockell, *J. Biol. Chem.*, **207**, 501 (1954).
(27) F. Sanger, in "Advances in Protein Chemistry," M. L. Anson, K. Bailey and J. T. Edsall, ed., Vol. 7, Academic Press, Inc., New York, N. Y., 1952, p. 22.
(28) S. J. Leach, *Revs. Pure Applied Chem.*, **3**, 25 (1953).
(29) M. W. Rees, *Biochem. J.*, **40**, 632 (1946).

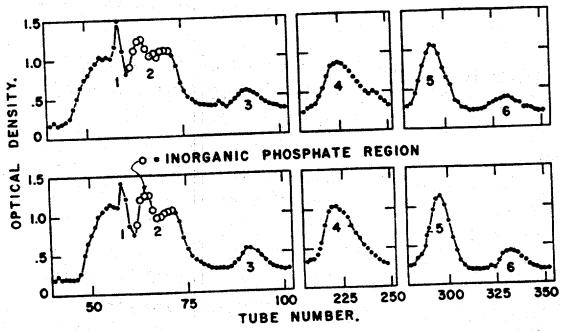

Fig. 1.—Chromatogram of the phosphopeptides in a partial hydrolysate of α-casein. Chromatography was carried out on a 3.8 × 100 cm. column of Dowex 50-X8 at 37.5° using 0.05 *N* hydrochloric acid as influent. The effluent was collected in 8-ml. fractions. Aliquots (0.5 ml.) were removed for analysis by the ninhydrin method. Each chromatogram was run on separate hydrolysates. The graph is corrected to an equivalent basis for variations in volume due to changes in the weight of drops during a run.

Paper electrophoresis showed only one of these fractions to be homogeneous. The other fractions contained up to four or more components. Fraction 5, which appeared to be homogeneous, had the same mobility as phosphoserine, but on complete hydrolysis it was found to contain aspartic acid and glutamic acid in addition to serine.

TABLE I

NITROGEN AND PHOSPHORUS COMPOSITION[a] OF PHOSPHOPEPTIDE FRACTIONS OBTAINED FROM PARTIAL ACID HYDROLYSIS OF α-CASEIN

(Fractions are numbered as in Fig. 1. The Dowex 50 column was charged with 286.0 mg. of nitrogen and 20.63 mg. of phosphorus.)

Fraction	Nitrogen, mg.	Organic phosphorus, mg.	Atomic ratio,[e] N/P
1	4.74	3.96	2.7
2	2.94	2.39[c]	2.7
3	0.96	0.73	2.9
4	2.74	1.70	3.6
5	2.43	2.26	2.4
6	0.85	0.50	3.8
Base line[b]	10.76	5.04	4.7
Total	25.42	16.58	
Recovery, %	8.9	90.7[d]	

[a] Values are corrected for aliquots taken from fractions for ninhydrin analysis based on average weight of fraction. [b] Includes all tubes of the chromatogram not taken for the fractions. [c] In addition this fraction contains 2.14 mg. of inorganic phosphorus which is equivalent to a conversion of 9.35% of the phosphorus of α-casein to inorganic phosphorus. [d] Includes the inorganic phosphorus in fraction 2. [e] The calculated atomic N/P ratio of α-casein is 34.4.

Rechromatography of Fractions.—Exploratory experiments with an anion-exchange column of Dowex 1-X10 in the hydrogen form, using dilute hydrochloric acid as influent, showed that only the last three fractions from the Dowex 50 column could be resolved into several subfractions. Attempts to resolve the first three fractions with various forms of Dowex 50 or Dowex 1 and 2 using a number of volatile influent solvents have not been successful.

For chromatography on Dowex 1-X10, fractions 4, 5 and 6 were evaporated to dryness in a rotary evaporator and then made to 25 ml. with 0.01 *N* hydrochloric acid. Twenty-four milliliters was applied to a 1.9 × 20 cm. column and the remainder was retained for the determination of recovery from the column in terms of optical density. The effluent was collected in 8.5-ml. fractions at 25 ml. per hour and 0.5-ml. aliquots were taken for analysis by the ninhydrin method. The chromatograms for fraction 4, 5 and 6 are shown in Figs. 2, 3 and 4, respectively. The per cent. of each subfraction shown in the figures is in terms of total optical density units eluted from the column. The average recovery from the column for each of the three fractions in these terms exceeded 96%.

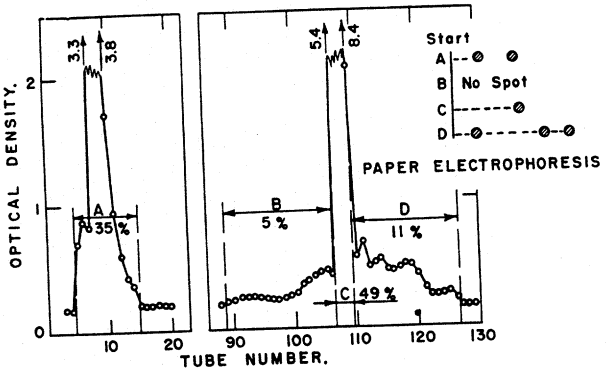

Fig. 2.—Chromatogram of fraction 4 obtained as shown in Fig. 1. Chromatography was carried out on a 1.9 × 20 cm. column of Dowex 1-X10 using 0.01 *N* hydrochloric acid as influent to tube 92 where a gradient with 0.1 *N* hydrochloric acid was used. The normality at subfraction C was 0.015. The effluent was collected in 8.5-ml. fractions and 0.50-ml. aliquots were removed for analysis by the ninhydrin method. Paper electrophoresis was performed as described in the text.

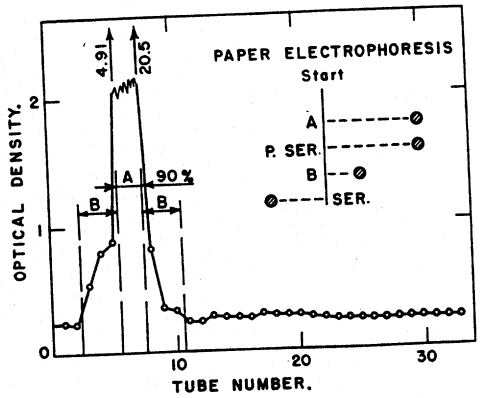

Fig. 3.—Chromatogram of fraction 5 obtained as shown in Fig. 1. Chromatography was carried out on 1.9 × 20 cm. column of Dowex 1-X10 using 0.01 *N* hydrochloric acid as influent. The effluent was collected in 8.5-ml. fractions and 0.50-ml. aliquots were removed for analysis by the ninhydrin method. Paper electrophoresis was performed as described in the text.

For paper electrophoresis, the subfractions were combined and concentrated *in vacuo* and made to the same optical density in order to obtain comparable concentrations. The electrophoretic results are also shown in Figs. 2, 3 and 4. The influent for the chromatography of fraction 4 shown in Fig. 2 was 0.01 *N* hydrochloric acid to tube No. 92 where a 500-ml. ground joint wash bottle was used as a mixing chamber for gradually increasing normality by addition of 0.1 *N* hydrochloric acid. The acidity of the effluent at subfraction C (Fig. 2) was 0.015 *N* as shown by titration. The results obtained by paper electrophoresis showed that only subfraction C was homogeneous; subfractions A and D contained a total of five components, and B was insensitive to the ninhydrin spray.[19]

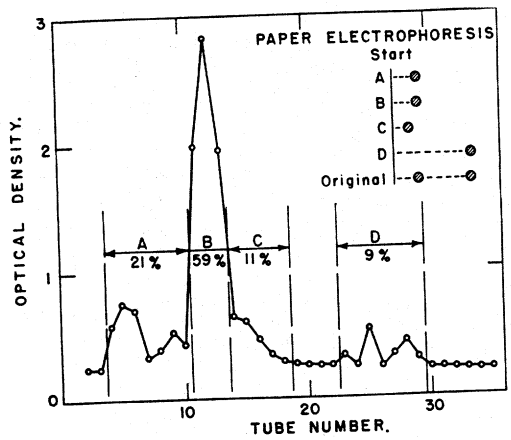

Fig. 4.—Chromatogram of fraction 6 obtained as shown in Fig. 1. Chromatography was carried out on 1.9 × 20 cm. column of Dowex 1-X10 using 0.01 *N* hydrochloric acid as influent. The effluent was collected in 8.5-ml. fractions and 0.50-ml. aliquots were removed for analysis by the ninhydrin method. Paper electrophoresis was performed as described in the text.

Fraction 5, which appeared to be homogeneous by paper electrophoresis, having the same electrophoretic mobility and behavior on Dowex 50-X8 as phosphoserine, was rechromatographed on Dowex 1-X10 with 0.01 *N* hydrochloric acid as influent solvent. The chromatogram of fraction 5 (Fig. 3) shows that 90% of the fraction is now concentrated in two tubes and that the fraction contained two electrophoretic components. The nature of the chromatogram indicates that complete resolution was not obtained. This was later confirmed when subfraction A was resolved on the Dowex 50-X8 column of Moore and Stein[16] for the determination of amino acids. Phosphoserine emerged without holdup at the front as reported by Moore and Stein,[30] followed by another peak separated by one tube having a low optical density. This was considered to be a satisfactory resolution in view of the high proportion of one component. On the basis of optical density and without correction for the color factor, which is unknown for the minor component, subfraction A contains 96.3% phosphoserine.

Chromatography of fraction 6 on Dowex 1-X10 with 0.01 *N* hydrochloric acid as influent solvent is shown in Fig. 4. The four subfractions as shown by paper electrophoresis contained three components. As judged by the nature of the

(30) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 893 (1954).

chromatogram, subfraction D should contain two components one of which may be insensitive to the ninhydrin spray; in subfraction A the chief component has apparently the same electrophoretic mobility as the component in subfraction B which contained 59% of the total material. Fraction 6, therefore, contains at least five components as determined by an analysis of its subfractions compared with only two components that were found by paper electrophoresis of the original material.

The character of the three chromatograms suggests that each of the main subfractions contained some impurity. This could not be established by either paper electrophoresis or paper chromatography, even when large amounts of samples were used for analysis. That the subfractions did contain up to about 10% of impurity was later confirmed when the amino acid compositions were determined.

The main subfraction of each fraction shown to be homogeneous by electrophoresis (4-C, Fig. 2; 5-A, Fig. 3; and 6-B, Fig. 4) was analyzed qualitatively for amino acid composition after complete hydrolysis. Each of the subfractions was found to contain glutamic acid, aspartic acid and serine, and subfraction 6-B also contained alanine. The only N-terminal amino acid that was found by paper chromatography studies on the hydrolyzed DNP subfractions was serine.

The resolution of fractions 1 and 2 (Fig. 1) into several subfractions with paper chromatography using butanol–acetic acid–water (5:4:1) suggested that chromatography on a cellulose column under similar conditions might be successful. Experiments with a 1 × 25 cm. cellulose column resolved each fraction into several ninhydrin-positive peaks. The first influent solvent was a butanol–acetic acid–water (10:1:2) system, which did not interfere with the ninhydrin determination, followed by 50% ethanol–water gradient and then by an aqueous hydrochloric acid gradient. Analysis of the subfractions obtained in large scale experiments with 3.8 × 25 cm. columns showed that only one of them (from fraction 2, designated 2-C) was electrophoretically homogeneous. This was eluted at the change-over to 50% ethanol–water and contained serine and glutamic acid.

Amino Acid Analyses of Subfractions.—The amino acid analyses of the main subfractions which were electrophoretically homogeneous are shown in Table II. The results are expressed in terms of the molar ratios of the constituent amino acids. Except as noted, the values in the table for 4-C were calculated from the data on the 140-hour hydrolysate since the 70-hour hydrolysis was not complete. For 5-A and 6-B the values are the average of the 70- and 140-hour hydrolysate values which agreed within experimental error. The values reported for subfraction 2-C were obtained on an hydrolysate which was complete after only 20 hours.

The amino acid chromatograms of some of the subfractions showed several ninhydrin peaks for which the amino acids could not be found or established by paper chromatography and are not included in Table II. For subfractions 4-C, 5-A and 6-B one of these peaks, less than 0.02 of a residue, appeared at the front of the chromatogram where phosphoserine is eluted. In control experiments, however, with a mixture of the amino acids and phosphoserine subjected to the same hydrolytic conditions, no new peaks appeared and the phosphoserine was hydrolyzed completely. The first peak, therefore, is due to an impurity which on hydrolysis yields an acid compound; the second peak, equivalent to less than 0.03 of a residue, appeared just before the serine peak with good resolution. This peak may well be due to threonine, although it could not be detected by paper chromatography presumably because of its low concentration. It is possible, therefore, that a peptide containing threonine constitutes a minor impurity in the subfractions.

TABLE II

AMINO ACID COMPOSITION OF ELECTROPHORETICALLY HOMOGENEOUS PHOSPHOPEPTIDE SUBFRACTIONS OBTAINED FROM PARTIAL ACID HYDROLYSIS OF α-CASEIN

(Results are expressed in terms of the molar ratios of the constituent amino acids.[a])

	Subfractions			
	4-C	5-A	6-B	2-C
Aspartic acid	**0.11**	0.03	0.06	..
Glutamic acid	**0.92**	0.07	0.12	0.14
Alanine	..	..	**1.08**	..
Serine (extrapolated[b])	0.73	0.86	0.93	0.96[e]
Serine (min.[c])	**1.00**	**1.00**	**1.00**	**1.00**
Phosphoserine[d]	..	1.02	..	..

[a] Based on the minimum serine content taken as unity. [b] Value obtained by straight-line extrapolation of data from 70- and 140-hour hydrolysis to zero time. [c] Calculated from the phosphorus content and based on the assumption that the phosphorus is present as the mono-ester of serine. [d] Determined on unhydrolyzed subfraction by chromatography on Dowex 50-X8 resin of Moore and Stein.[16] [e] Value is the sum of the serine and the ammonia formed by destruction of serine determined on a single hydrolysis with 6 *N* hydrochloric acid at 120° for 6 hours by chromatography on the Dowex 50-X8 resin.

The chromatogram of the hydrolysate of subfraction 4-C contained additional components eluted as an unresolved double peak in the region where glycine and alanine appear. This double peak, present to the extent of 0.38 of a residue in the 20-hour hydrolysate, was reduced to 0.14 of a residue in the 70-hour hydrolysate; no further reduction was obtained in the 140-hour hydrolysate. This is a sufficient quantity to be detected by paper chromatography, but the presence of glycine or alanine could not be established. The double peak, therefore, contains a peptide which is apparently resistant to 140-hour hydrolysis. That this peptide might contain valine is suggested by the appearance of a peak in the chromatogram of 0.08 of a residue where valine is expected to be eluted and by the known resistance to acid hydrolysis of peptides containing valine.[26,31]

The compositions of the individual subfractions in terms of integral number of amino acid residues per mole are given in boldface type in Table II. The method of analysis, however, is capable of revealing the presence of impurities in the subfractions at levels which are not detectable by paper

(31) E. L. Smith, A. Stockell and J. R. Kimmel, *J. Biol. Chem.*, **207**, 551 (1954).

electrophoresis or by paper chromatography of the hydrolyzed amino acids.

From an analysis of the data in Table II the composition of the subfraction as given in Table III can be calculated based on the following additional experimental evidence: (1) serine was the only amino acid that was found to be N-terminal in each of the subfractions (not determined for 2-C) by paper chromatography of the hydrolyzed DNP subfractions. Since the DNP phosphopeptides are resistant to hydrolysis, the long periods of heating required for complete hydrolysis cause an excessive destruction of serine, making the quantitative determination of N-terminal serine difficult. (2) The chain length of the main constituent of the subfractions was estimated by a determination of the ratio of the ninhydrin color value of the hydrolyzed as compared to the unhydrolyzed material. The ratio is of significant value when the chain length is small, and the amount of impurity of unknown chain length is low. Furthermore, this ratio can be used only for the estimation of short chain lengths with any degree of certainty, because the relative color value of serine and other dipeptides varies considerably as shown by Harris, *et al.*,[32] and Dowmont and Fruton.[33] These variations may lead to false conclusions, particularly for longer chain peptides. For the first three subfractions in Table III the experimentally determined ninhydrin ratio of the 70-hour hydrolysate to the unhydrolyzed is 2.0, 1.2 and 2.0, respectively. For subfraction 2-C using a 20-hour hydrolysate, the experimentally determined ratio is 1.8; however, due to the uncertainty of the relative color value of the unhydrolyzed peptide, this ratio is taken as 2. The value of 1.2 for subfraction 5-A can be accounted for by the presence of 10% of a tripeptide as an impurity which is consistent with the Van Slyke amino-nitrogen values.

The Van Slyke analysis on subfraction 4-C is of particular interest. This subfraction reacted with nitrous acid to yield all of its nitrogen as α-amino nitrogen under the usual conditions. The liberation of 2 moles of nitrogen with nitrous acid from γ-dipeptides of glutamic acid and other γ-glutamyl dipeptides was shown to occur by Sachs and Brand.[35,36] Since serine, however, was found to be the N-terminal amino acid of the dipeptide, a γ-glutamyl structure for the dipeptide is not possible. A mechanism for the liberation of peptide nitrogen from γ-glutamyl-peptides with nitrous acid was shown to parallel the formation of reaction products with a lactone structure by Sachs and Brand.[36] The same mechanism may be applicable for the phosphoserylglutamic acid dipeptide where the elements of water necessary for lactone formation are present in the phosphate radical.

TABLE III

COMPOSITION OF SUBFRACTIONS[a]

	% of constituents on mole basis			
	4-C	5-A	6-B	2-C
Pser. Glu	90			
Pser. Asp	10			
Pser.		90[c]		
Pser. (Asp, Glu)		10		
Pser. Ala			90	
Pser. (Ala, Glu, Asp)			10	
Pser. Pser.				75[d]
Glu. Pser.[b]				25[e]

[a] The abbreviations for amino acids and conventions for indicating their sequence are those of Sanger and Tuppy[34]; when the sequence of amino acids in a peptide is known, they are separated by periods, and when not known they are enclosed in parentheses and separated by commas. Additional abbreviations: Pser = O-phosphoserine. [b] This structure is assigned because the peptide Pser.Glu is resolved in another fraction from Dowex 50 (4-C). [c] On the basis of ninhydrin optical density, 96% of this subfraction is phosphoserine. [d] This value is 87% if the impurity in this subfraction is a tripeptide, such as (Glu, Pser.Glu) instead of a dipeptide. [e] Equivalent to 12% on molar basis and 10% on weight basis of glutamic acid as impurity in subfraction.

Discussion

The amount of destruction of serine found on hydrolyzing serine phosphopeptides as shown in Table IV is greater than has been reported for the destruction of serine in proteins. The values usually taken for the destruction of the hydroxy amino acids were established in the careful study by Rees[29] averaging 10% for serine and 5% for threonine after 24 hours in boiling 6 *N* hydrochloric acid. In the experiments by Hirs, *et al.*,[37] the loss of serine was 16% and that of threonine was 8% when either ribonuclease or a synthetic mixture of amino acids corresponding to an hydrolysate of serum albumin was heated for 20 hours with 6 *N* hydrochloric acid in sealed tubes at 110°. Good recoveries, however, were obtained by extrapolation of the analytical results to zero time. Gordon and Ziegler[38] found that serine and threonine were completely stable in the hydrolysis of α-lactalbumin at 120° even when the protein was hydrolyzed for 140 hours. The results in Table IV show that the lability of serine is apparently dependent in some degree on the amino acid with which it is associated. The value of 64.6% recovery for the 70-hour hydrolysate of subfraction 5-A, which is essentially phosphoserine, is comparable to the value of 61.0 obtained by Hirs and associates[37] for the recovery of serine from a 70-hour hydrolysate at 110° of a synthetic mixture

TABLE IV

RECOVERY OF SERINE FROM SUBFRACTIONS HEATED WITH 6 *N* HCl AT 120°

Subfraction	% Recovery[a] Time of hydrolysis 70 hr.	140 hr.	By extrapolation to zero time
4-C[b]	55.5	38.3	73
5-A	64.6	42.0	87
6-B	75.2	54.5	93

[a] Calculated from the phosphorus content and based on the assumption that all of the serine is phosphorylated and is present as the monoester. [b] A 20-hour hydrolysate contained phosphoserine.

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(35) H. Sachs and E. Brand, THIS JOURNAL, **75**, 4608 (1953).

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(37) C. H. W. Hirs, W. H. Stein and S. Moore, *J. Biol. Chem.*, **211**, 941 (1954).

(38) W. G. Gordon and J. Ziegler, *Arch. Biochem. Biophys.*, **57**, 80 (1955).

corresponding in composition to a hydrolysate of bovine serum albumin. This suggests that the phosphate itself has no appreciable effect on the hydrolytic destruction of serine. It is apparent from the last column of Table IV that a correction for losses of serine by extrapolation of the results to zero time, assuming first-order kinetics for the decomposition, would lead to considerable error.

Phosphoserylglutamic acid was first isolated by Levene and Hill[40] from an enzymatic digest of casein. de Verdier[39] has reported the presence of phosphoserylglutamic and phosphoserylalanine and several other peptides of phosphoserine from acid digests of casein but was not able to establish that serine was N-terminal.

(39) C. H. de Verdier, *Acta Chem. Scand.*, 8, 1302 (1954).

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